Claims 7 and 8 are pending. These claims are amended to delete the terms "substantially" and the abbreviation "HSV" as suggested in the Office Action, to further clarify what is claimed. New claim 16 is added and corresponds to claim 11 from the copending application, except that the language objected to in claim 11 of the copending application is absent. No new matter is added. In light of the above amendments and the following remarks, reconsideration and further examination are respectfully requested.

I. Informalities

A. The oath or declaration is indicated to be defective, because the copy of the oath is of such poor quality as to render the oath unreadable. In this regard, applicants submit herewith a copy of a substitute oath, which was filed during the prosecution of the parent application (Serial No. 07/691,728). This declaration is believed to be in compliance with 37 C.F.R. 1.67. Thus, withdrawal of this objection is believed to be merited, and is respectfully requested.

B. The Office Action states that an amendment must be made to the brief description of drawings to reflect the corrections of the drawings required in the PTO Form 948. As indicated, applicants have made the requested amendment. Thus, withdrawal of this rejection is believed to be merited and is respectfully requested.

II. Obvious Type Double Patenting

Claims 7 and 8 are provisionally rejected under the judicially created doctrine of obvious type double patenting as being unpatentable over claim 11 of copending application Serial No. 08/475,064.

In this regard, applicants have amended the present application to include new claim 16, which corresponds to claim 11 from the copending application, except that the language objected to in claim 11 of the copending application is absent. Applicants have done this, because as the Examiner points out, the original restriction requirement separating the subject matter of claims 7-8 from that of claim 11 was withdrawn pursuant to applicants' arguments submitted in the parent application. These arguments are believed to be applicable in the present

situation such that no restriction requirement separating these claims should be issued in the present application. Furthermore, any such restriction requirement would be entirely inconsistent with the present obvious-type double patenting rejection. Upon indication from the Patent Office that claim 11 will be allowed in the present application, applicants will cancel claim 11 from copending application Serial No. 08/475,064. Thus, provisional withdrawal of this rejection is believed to be merited and is respectfully requested.

III. Rejections Under 35 U.S.C. § 112, second paragraph

Claims 7-8 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the invention. The Office Action further states the following:

> Claims 7-8 are vague and indefinite in the recitation "substantially pure" since it is entirely unclear what level of purification would constitute a "substantially pure" sample. applicant has defined "substantially pure" in the specification (see page 7, first full paragraph) but applicant's definition is equally vague and indefinite and fails to proscribe the metes and bounds of claims 7-8. Amendment of claims 7-8 to delete "substantially" would obviate this rejection. Claims 7-8 are further vague and indefinite in the recitation "HSV" since abbreviations should be avoided in the claims and

> it is unclear what HSV would correspond to. Amendment of claims 7-8 to recite "Herpes Simplex Virus" would obviate this rejection.

(April 30, 1996 Office Action, page 4, lines 20-31)

In this regard, applicants have deleted the term "substantially" such that the claims recite "pure herpes simplex virus gG-1 antigen" and "pure herpes simplex virus gG-2 antigen." This amendment addresses the above issue and further clarifies the claim by using the full term instead of the abbreviation "HSV." Thus, withdrawal of this rejection is believed to be merited and is respectfully requested.

IV. Rejections Under 35 U.S.C. § 102 and § 103

The present invention is rejected as either anticipated or obvious over Lee et al. (AW or AA1) alone or in combination with Luckow et al. or Matsuura et al.

The present invention relates to novel recombinant herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) envelope glycoproteins (gG-1 and gG-2, respectively) produced by a novel process using a novel recombinant baculovirus. The novel recombinant baculovirus is produced by a novel process using a

novel plasmid, pAcDSM.

The novel plasmid, pAcDSM, is engineered to receive a foreign gene precisely at the translation initiation codon of the polyhedron gene, without missing any nucleotide present in the native 5' nontranslated leader sequence and translation initiation codon and without introducing any extraneous nucleotides at the initiation codon site. This plasmid is a baculovirus transfer vector that is used to insert a foreign gene into the baculovirus *Autographa californica* nuclear polyhedrosis virus.

The novel recombinant baculovirus is produced by recombining sequences from pAcDSM with a foreign gene (e.g., HSV gG-1 and gG-2) and transfected into a baculovirus to produce a recombinant baculovirus. This baculovirus has the full 5' nontranslated leader sequence of the polyhedron gene joined to the coding region of a foreign gene precisely at the translation initiation codon of the native polyhedron gene, and neither omits any nucleotide from the initiation codon nor introduces any extraneous nucleotides at the initiation codon site. This recombinant baculovirus is capable of highly efficient transcription and translation of foreign genes and expression of

novel proteins within a baculovirus system.

The cited prior art does not teach the claimed proteins or how to obtain the present novel proteins.

A. Rejections Under 35 U.S.C § 102

Claims 7-8 are rejected as anticipated by, or in the alternative, as obvious over either Lee et al. (A) or Lee et al. (A). In this regard the Office Action further states the following:

> Applicant's claimed invention are directed to products of recombinantly expressed gG-1 or gG-2 antigens of HSV. Applicant's claims constitute Product-by-Process type claims. In a product-by process claim, the process of producing the product is given no patentable weight. *In re Brown,* 173 USPQ 685, 688 (CCPA 1972). Lee et al.(AW) teaches a pure HSV gG-1 antigen immunoaffinity purified by mouse monoclonal H1379-2 antibodies(see page 112, second full paragraph). Further, Lee et al. (AA1) teaches a pure HSV gG-2 antigen immunoaffinity purified by mouse monoclonal H966 antibodies (see page 641, second column, last paragraph, page 642, first column, first full paragraph and page 643, first column, fourth full paragraph). Since the Patent Office does not have the facilities for examining and comparing applicants' composition with the compositions of the prior art references, the burden is upon applicants to show an unobvious distinction between the material, structural and functional characteristics of the claimed composition and the compositions of the prior art. See In re Best, 562 F.2d 1252, 195 U.S.P.Q. 430 (CCPA 1977).

(April 30, 1996 Office Action, page 6, second sentence - end of that paragraph)

Applicants first point of response is that the claimed proteins differ from those of Lee et al. because the claimed proteins are expressed in a baculovirus insect cell system, whereas the proteins of Lee et al. are expressed in mammalian cells. Because of the well recognized differences in post translation processing of proteins in mammalian vs. bacteria vs. insect cells, there is no doubt that the claimed proteins differ from those of Lee et al. That the proteins produced in mammalian cells and insect cells are invariably different from each other in one or more ways is shown in Figs. 3C and 4D of Sanchez-Martinez and Pellett (*Virology* 182:229-238 (1991) attached as Exhibit 1) and Figs. 3E and 4D of the present application. These figures show the differences in the baculovirus-expressed and infected mammalian cell-expressed gG-1 and gG-2. Thus, because the claimed proteins differ from the proteins of Lee et al., the disclosures of Lee et al. cannot anticipate the claims to the present proteins. Thus, withdrawal of this rejection is believed to be merited and is respectfully requested.

Furthermore, there are differences in the proteins produced by the two baculovirus expression systems compared by Sanchez-Martinez and Pellett. In describing an experiment comparing the major reacting bands in immunoblots of AcDSMgG-1- and Ac373'gG-1-expressed proteins, the reference states that "[e]xpression of the recombinant gG-1s differed in two respects.... (ii) In extracts of Sf9 cells infected with AcDSMgG-1, 42K reacted more that 37K. In extracts of Sf9 cells infected with Ac373'gG-1, the opposite was true, with 42K being very faint" (page 233, left column). In describing an experiment comparing the major reacting bands in slot blots of AcDSMgG-1- and Ac373'gG-1-expressed proteins, the reference notes the same difference in the expressed proteins and concludes that "the difference in processing of gG-1 may be due to differences between the transfer vectors" (page 233, right column). This teaches that not only are the baculovirus- and infected mammalian cell-expressed proteins different from each other, but also that the present baculovirus-expressed proteins differ from the same protein produced in other baculovirus expression systems based on the way they are produced.

The present recombinant baculovirus vector used to express the present proteins produce differences (e.g., through

postranslational processing) in the resulting proteins. Because these differences in the claimed proteins compared to other baculovirus-expressed proteins were not suggested in the prior art, the present protein could not have been expected based on disclosure of other baculovirus vectors. As a result, even if the process steps are not considered, the proteins are different in a way that could not be expected prior to the present teaching of the novel AcDSMgG-1 and AcDSMgG-2-produced proteins. Thus, a finding of novelty and unobviousness is beleived to be merited and is respectfully requested.

B. Rejections Under 35 U.S.C § 103

Claims 7-8 are rejected under 35 U.S.C. § 103, as being unpatentable over Lee et al. (AA1) or Lee et al. (AW) in view of Luckow et al. (AO) or Matsuura et al. (AP). In this regard, the Office Action further states the following:

> Lee et al. (AA1) and Lee et al. (AW) disclose purified gG-2 and gG-1 respectively as discussed above. Lee et al. (AA1) and Lee et al. (AW) do not disclose the production of gG-1 or gG-2 in a baculovirus expression system. Luckow et al. discloses the development of baculovirus expressions systems and the advantages of using such systems for "the very abundant expression of recombinant proteins, which are in many cases, antigenically, immunologically, and functionally similar to their authentic counterparts" (see page 47, first column, first full paragraph). Luckow et al. also discloses many of the vectors suitable

> for baculovirus expression systems and the importance of particular leader sequences upstream from the polyhedrin gene ATG and their impact on protein production (see page 51, first column, last paragraph). Similarly, Matsuura et al. also discloses the uses and advantages of the baculovirus expression system and teaches "that the immediate 5' upstream sequences are important for high level expression" (see page 1234, Figure 1 and see page 1247, second full paragraph). Matsuura et al, also discloses vector pAcRP18 which has the "51 nontranslated leader sequence of the polyhedrin gene joined to the coding region of a foreign gene precisely at the translation initiation codon of the polyhedrin gene" of the claimed inventions. Neither Luckow et al. or Matsuura et al. teach the expression of gG-1 or gG-2 peptides in baculovirus expression systems. However, the level of ordinary skill in the genetic engineering art is exceptionally high and, absent convincing objective evidence to the contrary, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the claimed invention was made to express the gG-2 protein of Lee et al. (AA1) or the gG-1 protein of Lee et al. (AW) in the baculovirus expression system of Luckow et al. or Matsuura et al. for the expected benefit of obtaining high levels of expression of gG-1 and gG-2 proteins. One of ordinary skill in the art would have been motivated to express the proteins in a baculovirus system since Luckow et al. and Matsuura et al. disclose the numerous advantages of baculovirus expressions systems, the importance of retaining an intact 51 nontranslated leader sequence of the polyhedrin gene and one of ordinary skill would have had a reasonable expectation of success since Luckow et al. and Matsuura et al. both establish that the baculovirus system produces significant amounts of peptides and that such peptides "are in many cases, antigenically, immunologically, and functionally similar to their authentic counterparts."

(April 30, 1996 Office Action, second line of the second paragraph of page 6 - sentence bridging pages 7 and 8).

Before responding to the specifics of the above rejection, applicants again point out that the present specification and Exhibit 1 show that the presently claimed proteins differ from any predicted baculovirus-expressed gG-1 or gG-2, because of the unique and unpredicted processing differences that result from the present expression system. Thus, the proteins (products) themselves are unobvious over any previous mammalian-expressed or any predicted baculovirus-expressed protein. Therefore, withdrawal of this rejection on this basis alone is believed to be merited and is respectfully requested.

The Examiner's comments above are directed to the novelty and unobviousness of applicants' process of producing applicants' claimed proteins. For the reasons stated above and following, the combination of references cited by the Examiner does not teach one skilled in the art what the claimed proteins are or how to make them.

As asserted in the Office Action, there are references showing (1) purified mammalian cell produced HSV-1 gG and HSV-2 gG (Lee et al., AW and AA1); (2) the general usefulness of the baculovirus-insect cell expression system and the potential

importance of leader sequences upstream from the ATG of the polyhedrin gene (Luckow et al., AO); and (3) the potential importance of the immediate 5' upstream nontranslated polyhedrin leader sequences from AcNPV in improving the efficiency of expression in an AcNPV-insect cell expression system (Matsuura et al.). The Examiner asserts that the present purified proteins are obvious in view of the previous disclosure of the suggested importance of the 5' nontranslated sequence in baculovirus expression systems and the existence of purified HSV gG-1 and gG-2 protein.

Contrary to the implication of the Office Action, none of the prior art references accomplish what Matsuura et al. and Luckow et al. suggest is important. While Matsuura et al. does disclose 12 plasmids with different 5' nontranslated leader sequences, none precisely replicate the 5' nontranslated sequence and translation initiation codon of the wild-type baculovirus. The present expression method does. pAcRP18, described by Matsuura et al. as having "51 nontranslated leader sequence of the polyhedrin gene joined to the coding region of a foreign gene precisely at the translation initiation codon of the polyhedrin gene," does not faithfully reproduce the translation initiation codon. Rather, pAcRP18 does not include the translation

initiation codon. The following provides a comparison of the most relevant sequence of Matsuura et al., the wild-type and the present plasmid:

-10 +1	
ACCTATAAATATG	wild type (as shown in Fig. 1 of Matsuura et al.)
ACCTATAAATcggatc	pAcYM1 (as shown in Fig. 1 of Matsuura et al.)
ACCTATAAATATG	pAcDSM (Applicants')

The five nucleotide difference shown is the minimum number of extraneous nucleotides compared to the native sequence, assuming digestion with BamH1 and fill-in of the overhanging nucleotides. In contrast, the plasmid pAcDSM and the recombinant baculovirus exactly mimic the polyhedrin regulatory sequences. This provides advantages in transcriptional and translational efficiency not provided by Matsuura et al. An important element of this invention is that the steps taken by appellants allows insertion of genes into the vector with no extraneous nucleotides. Thus, the prior art did not teach a recombinant baculovirus expression system with the same features or advantages as the present system.

The cited references must provide motivation to generate the claimed invention in order to render that invention obvious. However, providing an incentive to try to make the invention, absent a reasonable expectation of successfully obtaining the claimed invention is not sufficient to render an invention obvious (Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 231 USPQ2d 81 (Fed. Cir. 1986)). In this regard, applicants concede that Luckow et al. and Matsuura et al. provide an incentive to obtain a baculovirus transfer vector that maintains the 5′ nontranslated sequence and start initiation codon of the wild-type polyhedron promoter. What is missing from Luckow et al. and Matsuura et al. and the other cited references is the technical guidance that would provide a reasonable expectation of accomplishing this goal. Thus, the claimed novel proteins made by the process using the present plasmid and recombinant baculovirus are not obvious.

Matsuura et al. do not teach or suggest a way to accomplish the solution provided by the invention. In fact, Matsuura et al. disclose 12 plasmids with different 5′ nontranslated leader sequences, but none that succeeded in precisely replicating the 5′ nontranslated sequence and translation initiation codon of the wild-type baculovirus. Thus,

Matsuura et al., after disclosing an incentive to obtain the present invention and numerous examples of failed attempts, could not provide a way to make pAcDSM, because, until the present invention, it was not obvious how to accomplish that goal. Moreover, the very reference which the Examiner alleges to provide motivation could not overcome the scientific hurdles to produce that which was desired and which applicants have provided.

Thus, the mere suggestion by Luckow et al. and Matsuura et al. of the problem to be solved, namely constructing a plasmid that faithfully reproduces a 5' nontranslated polyhedron leader sequence, is nothing more than a wish or a desire for a result. Such a desire or wish is legally insufficient without a disclosure of the means to carry out the invention. See Hybritech, Inc. v. Monoclonal Antibodies, Inc., 801 F.2d 1367, 231 USPQ2d 81 (Fed. Cir. 1986) (Invitations to try to accomplish something do not show obviousness since they "do not suggest how that end might be accomplished.").

The case law is clear that an Examiner cannot use hindsight based on the applicant's own teaching of the invention to fill in the gaps in the prior art. See In re Gorman, 933 F.2d

982, 18 USPQ2d 1885, 1888 (Fed. Cir. 1991) ("[I]t is impermissible ... simply to engage in a hindsight reconstruction of the claimed invention, using the applicants' structure as a template in selecting elements from references to fill in the gaps.").

The steps taken by applicants to arrive at the instant plasmid required not only an understanding of the importance of the 5' nontranslated leader sequence, but also the conception of a way to conserve that sequence while all others, including Luckow et al. and Matsuura et al., had failed.

The Examiner has done no more than to state that there existed a general motivation and that the ordinary level of skill in genetic engineering is high, and conclude that the invention is obvious. Such a rationale taken to its logical conclusion would render every invention in molecular biology obvious. The Examiner is forced to make this sweeping conclusion because none of the cited references teach how to accomplish what applicants have accomplished. The case law is very clear that a teaching to form a basis of an obvious rejection must be in the cited references. See In re Dow Chemical Co., 837 F.2d 469, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988) ("The consistent criterion for

determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success, viewed in the light of the prior art ... Both the suggestion and the expectation of success must be found in the prior art, not in the applicant's disclosure."). See also Northern Telecom, Inc. v. DataPoint Corp., 908 F.2d 931, 15 USPQ2d. 1321, 1344 (Fed. Cir. 1990) ("Whether the changes from the prior art are 'minor', ... the changes must be evaluated in terms of the whole invention, including whether the prior art provides any teaching or suggestion to one of ordinary skill in the art to make the changes that would produce the patentees [invention]." Such a specific teaching is simply not to be found in the cited references.

Undue experimentation would be required to make the claimed invention and, therefore, the invention cannot be obvious. The amount of experimentation required to make the invention should be considered to make a case of obviousness. In re Dow Chemical Co., 5 USPQ2d 1529 (Fed. Cir. 1988); Westnofer USA v. Whole Life Co., Inc., 3 USPQ2d 1352, 1354, 1355 (D.C.D. Massachusetts, 1987) ("The significant resources devoted to developing this ... [invention] ... and the fact that development

extended over three years support an inference that this invention was not obvious. The substantial efforts made by the inventors also indicate that the invention was not easily made by one skilled in the art."). Also, by inference from the enablement context, it is clear that an invention should not be considered obvious if the amount of experimentation required to obtain it was undue experimentation. Hybritech, Inc. v. Monoclonal Antibodies, Inc., 231 USPQ2d 81, 94 (Fed. Cir. 1986); Amgen Inc. v. Chugai Pharmaceutical Co., Ltd., 18 USPQ2d 1016, 1023 (Fed. Cir. 1991) (In supporting the lower court's conclusion that the subject invention was unobvious, the court noted the testimony of an expert to the effect that "it would have been 'difficult' to find the gene." The lower court had found that "no one had successfully screened a genomic library using fully degenerate probes of such high redundancy as the probes used by [the inventor]").

The conception of the present invention was more than routine experimentation both in terms of the problem to be solved and in terms of the length of time required to solve the problem. One postdoctoral fellow worked full-time on the conception of pAcDSM for over nine months, while the Chief of the Herpesvirus Section of the Division of Viral Diseases at the Centers for

Disease Control and Prevention worked part-time to solve the problems posed by prior art efforts to express proteins for approximately one and one-half years.

Since the early years of the use of the polyhedron promoter and especially since the articulation by Matsuura et al. in 1987 of the purported advantages of the present invention, the need for the present invention has clearly been felt in the art. In the rapidly expanding commercial and scientific fields of recombinant proteins, given the recognition of the importance of the molecular biological tool provided by the present invention during the many years prior to the present invention, this need was long felt.

The many differences between the plasmids disclosed by Matsuura et al. and Luckow et al. and the pAcDSM used to produce the presently claimed proteins, along with the complex steps required to obtain pAcDSM, are schematically depicted in Exhibit A attached hereto.

Because no recombinant baculovirus was known in the art that could accomplish what the present invention accomplishes, and because the necessary steps required to advance beyond the

prior art baculoviruses to obtain the present recombinant baculovirus are not suggested in the prior art, the present baculoviruses containing particular foreign genes and the genes expressed thereby are also not suggested by the prior art. Therefore, the Examiner has not met the burden of showing that the recombinant HSV gG-1 and gG-2 proteins of claims 7 and 8 are obvious.

Moreover, the very tools available to applicants were available to Luckow et al. and Matsuura et al., yet, even though they recognized the desirability of applicants' claimed invention, they could not achieve it. Further, despite Matsuura et al.'s suggestion of the desirability of the claimed invention in 1987 and the molecular biology tools being available to the skilled artisan, none could make applicants' claimed invention.

The present process and products provide improved results over the plasmids, methods and proteins of the cited prior art.

Particularly, the applicants' application describes a slot blot analysis in which the intensity of the reaction of gG-1 protein and a monoclonal antibody specific for gG-1 was four-fold

greater in extracts from cells infected with AcDSMgG-1 (an example of a recombinant baculovirus of Claim 5) than with Ac373'gG-1 (specification page 19, lines 7-15; and Fig. 3B). Thus, it can be seen that the present recombinant baculovirus and method of producing pure protein (claim 15) provide higher levels of expression of gG-1 than a recombinant baculovirus, Ac373'gG-1, derived from pAc373 (Smith et al.). The proteins of the invention are qualitatively superior, because they are produced using the present recombinant baculovirus. For example, the invention provides glycosylated gG-1 at a higher ratio than nonglycosylated precursor protein (page 18, lines 20-24; page 25, lines 20-26), thus, more closely resembling the characteristics of the native HSV-1 gG-1 protein. Not only is more of the protein produced by the present method, but it is more efficiently post-translationally processed than the gG-1 produced using a recombinant baculovirus, Ac373'gG-1, derived from pAc373 of Smith et al. Thus, the claimed invention provides advantages in terms of both of quantity and quality not provided by the baculovirus expression systems provided by Matsuura et al. or others. Given these improvements, Matsuura et al. and Luckow et al. would have described applicants' constructs had they been obvious at the time those references were published.

As indicated above, Matsuura et al. and Luckow et al. neither provide a recombinant baculovirus capable of accomplishing what the present method accomplishes nor suggests how to obtain such a recombinant baculovirus. Thus, while it may be concluded that these references provide an incentive to accomplish what the present inventors have accomplished, it cannot be said that either reference provides a reasonable expectation of obtaining it. Thus, the Examiner has not met the burden of showing that the claimed invention is obvious.

The subject invention provides a key advancement in the production of HSV gG-1 and gG-2 proteins, particularly by the use of a novel baculovirus-insect cell expression system. The prior art did not suggest the advantages in the proteins produced according to applicants' method or the extensive experimentation (e.g., the creation of V78) required to create the present proteins. The present invention provides expressed proteins heretofore unavailable for either commercial or research applications.

In light of the above arguments, applicants request the withdrawal of the Examiner's rejection of the pending claims.

No fee is believed due; however, the Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: BOX NON-FEE AMENDMENT, Assistant Commissioner of Patents, Washington, D.C. 20231, on this 30th day of July, 1996.

Gwendolyn D. Spratt 7-30-96

Gwendolyn D. Spratt Date

VIROLOGY 182, 229–238 (1991)

Expression of HSV-1 and HSV-2 Glycoprotein G in Insect Cells by Using a Novel Baculovirus Expression Vector

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Received December 3, 1990; accepted January 15, 1991

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) glycoprotein G (gG-1 and gG-2) were expressed in insect cells from recombinant baculoviruses (AcDSMgG-1 and AcDSMgG-2, respectively) constructed using a novel baculovirus transfer vector, pAcDSM. This vector allows the coding region of a foreign gene to be precisely linked to the baculovirus polyhedrin gene at the translation initiation site and retains the native polyhedrin translation initiation environment. Fourfold more gG-1, with a higher ratio of glycosylated to unglycosylated product, was produced by AcDSMgG-1 than by Ac373'gG-1, a recombinant baculovirus which differs from AcDSMgG-1 by the presence of 21 extraneous nucleotides in the 5' nontranslated sequence. gG-1 and gG-2 expressed in recombinant baculovirus-infected insect cells undergo cotranslational N-linked glycosylation, but the overall processing of the proteins differs from that observed in HSV-1- or HSV-2-infected cells. Despite these differences, baculovirus-expressed gG-1 and gG-2 were recognized in a HSV type-specific manner by human serum specimens.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are genetically very similar (Kieff *et al.*, 1972; Ludwig *et al.*, 1972), resulting in extensive antigenic cross-reactivity (reviewed by Nahmias and Dowdle, 1968; and Honess and Watson, 1977). This cross-reactivity has resulted in many serologic tests for distinguishing prior infection with HSV-1 from that with HSV-2 being laborious as well as frequently inconclusive, particularly with specimens containing antibodies to both viruses (Nahmias *et al.*, 1970).

The identification of glycoprotein G as a type-specific antigen (gG-1) (Ackermann *et al.*, 1986; Richman *et al.*, 1986) (and gG-2) (Roizman *et al.*, 1984; Marsden *et al.*, 1984) allowed the development of accurate type-specific serologic assays (Lee *et al.*, 1985, 1986; Nahmias *et al.*, 1986; Ashley *et al.*, 1988). Studies using these assays have clearly demonstrated the accuracy that can be obtained using reactivity with gG-1 and gG-2 as the basis for the assay, but because of difficulties in preparing and standardizing reagents, the tests have been performed routinely in few laboratories. To obtain abundant supplies of well-characterized antigen for use in serologic tests and in studies of the host immune response to gG, and to bypass large-scale culturing of pathogenic agents, we have expressed gG-1 and gG-2 in the baculovirus expression system (reviewed by Luckow and Summers, 1988).

Although high levels of gene expression have been obtained using the baculovirus system, in only a few cases has the level of gene expression approached that of native polyhedrin. Kozak (1981) has shown that sequences immediately surrounding the translation initiation site can have a profound effect on the efficiency of translation initiation. We hypothesized that at least some of the differences in the level of gene expression between native polyhedrin and foreign genes inserted in its place may be due to missing or extraneous nucleotides in the vicinity of the translation initiation codon in vectors currently in use (Smith *et al.*, 1985; Matsuura *et al.*, 1987; Luckow and Summers, 1989). Thus the most efficient expression of a foreign gene in this system might occur if the native polyhedrin sequences controlling regulation of transcription and translation embedded in the 5' nontranslated leader sequence were unaltered and joined to the coding region of the foreign gene precisely at the translation initiation site, with no missing or extraneous nucleotides.

We describe in this report (i) the creation of a baculovirus transfer vector that fulfills the above requirements, (ii) its application for the construction of recombinant baculoviruses expressing gG-1 and gG-2 in insect cells, and (iii) the characterization and type specificity for HSV antibodies in human serum specimens of baculovirus-expressed gG-1 and gG-2.

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0042-6822/91 $3.00

MATERIALS AND METHODS

Cells and viruses

Wild-type and recombinant AcNPV were grown and assayed in a continuous ovarian cell line (Sf9) derived from *Spodoptera frugiperda* (fall armyworm), as previously described (Summers and Smith, 1987). Sf9 cells (ATCC No. CRL 1711) were obtained from the American Type Culture Collection (Rockville, MD). Wild-type AcNPV and the gene transfer vector pAc373 (Smith *et al.*, 1985) were obtained from Dr. Max Summers, Texas A&M University (College Station, TX). HSV-1(F) and HSV-2(G) (Ejercito *et al.*, 1968) were obtained from Dr. Bernard Roizman, University of Chicago (Chicago, IL) and grown and propagated as described (Morse *et al.*, 1977). Monoclonal antibodies specific for gG-1 (H1379) (Lee *et al.*, 1986) and specific for gG-2 (H1206) (Lee *et al.*, 1985) were obtained from Dr. Lenore Pereira, University of California, San Francisco (San Francisco, CA).

DNA manipulations

DNA manipulations were carried out essentially as described (Maniatis *et al.*, 1982). Restriction endonucleases and T4 DNA polymerase were purchased from New England BioLabs (Beverly, MA), mung bean exonuclease from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), and T4 DNA ligase from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Oligonucleotides were synthesized in a Model 380 DNA synthesizer from Applied Biosystems (Foster City, CA).

Construction of modified gene transfer vectors

pPP-2. pPP-1 was constructed by digesting pUC8 (Vieira and Messing, 1982) with *Nar*I and filling in the overhanging 5′ nucleotides using T4 DNA polymerase, followed by self-ligation, resulting in the elimination of the *Nar*I site. A synthetic oligoduplex AB (Fig. 1A) was ligated between the *Eco*RI and the *Hin*dIII sites of pPP-1, to obtain pPP-2. This vector can be used to construct gene fusions at any of the three reading frames by using blunt-end ligations at the appropriate site. The fused gene would then be removed from the vector by digestion at the flanking *Eco*RI and *Hin*dIII sites and inserted into a baculovirus transfer vector such as pAc373 at the desired site, using blunt ends if necessary.

pAcDSM. pAc373 was digested to completion with *Sal*I and *Kpn*I. The DNA fragment spanning the region between 3.18 and 4.43 kilobase pairs (kbp) in the coordinate system of Summers and Smith (1987) was purified from agarose and inserted into pUC19 (Norrander *et al.*, 1983) that had previously been digested with the same enzymes. The resulting plasmid (pDM1) was linearized by digestion with *Ava*I. After PEG precipitation (Sadhu and Gedamu, 1988), 5′ overhanging nucleotides were removed by digestion with 150 units of mung bean exonuclease/μg of DNA to obtain blunt ends, followed by *Kpn*I digestion. A synthetic oligoduplex, V78 (Fig. 1A), was inserted between the *Kpn*I site and the blunt end (nucleotide −9 of the 5′ leader sequence of the polyhedrin gene) of pDM1 to obtain pDM2. The fidelity of the construct was checked by nucleotide sequencing using primers flanking the cloned fragment. pDM2 was digested with *Eco*RV and *Kpn*I. The resulting 118-bp fragment was ligated to pAc373 previously digested with the same enzymes to obtain the transfer vector pAcDSM.

In order to use pAcDSM, it is digested with *Pst*I, treated with T4 DNA polymerase to trim the 3′ overhanging nucleotides to a blunt-ended C at position −9 of the polyhedrin 5′ nontranslated leader sequence, and then digested at another restriction site in the polylinker using an enzyme which generates a cohesive terminus, allowing for efficient directional insertion of a foreign gene (Fig. 1B). The foreign gene is modified for compatibility with the vector by assembling a segment of DNA containing, from 5′ to 3′, the nucleotides −8 to −1 of the 5′ nontranslated leader sequence of the polyhedrin gene, the translation initiation codon, the coding region of the foreign gene, the downstream region through the polyadenylation signal, and a unique restriction site compatible with one in the transfer vector's polylinker. Segments of DNA containing these features can be constructed by using standard cloning techniques and synthetic oligonucleotides as was done here, by total synthesis using long synthetic oligonucleotides, or by using the polymerase chain reaction (Saiki *et al.*, 1985) with appropriately tailed primers. When the modified segment of DNA is inserted into pAcDSM prepared as described above, the C in position −9 of the polyhedrin 5′ nontranslated leader sequence is linked to the first nucleotide of the modified gene (C in position −8), regenerating the 5′ leader sequence of the polyhedrin gene.

Cloning gG-1 and gG-2 into baculovirus transfer vectors

For convenience, the nucleotide sequence coordinates reported for the genomic sequence of HSV-1 strain 17 (McGeoch *et al.*, 1988) and for the *Hin*dIII L fragment of HSV-2 strain HG52 (McGeoch *et al.*, 1987) are used throughout this paper. HSV-1 strain F (HSV-1(F)) and HSV-2 strain G (HSV-2(G)) DNAs were separately digested to completion with *Bam*HI and shotgun cloned into pUC19. Plasmids carrying HSV-1(F) *Bam*HI

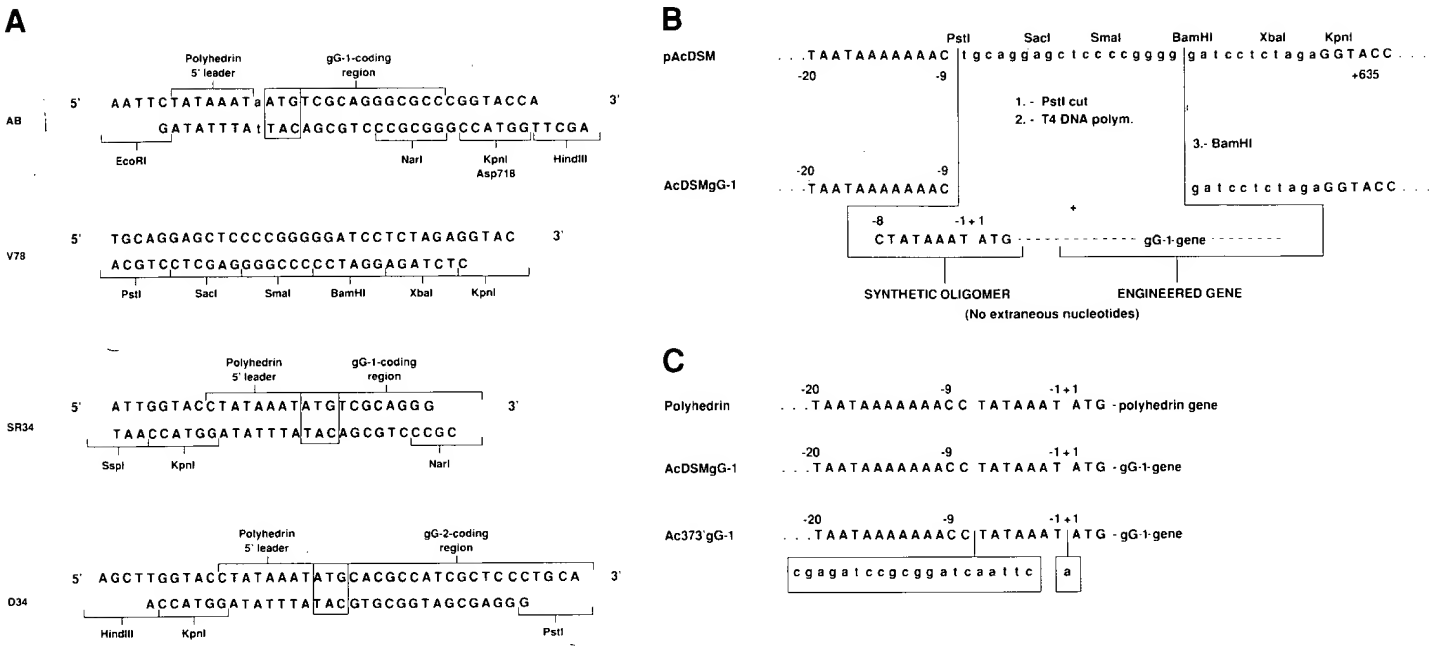

FIG. 1. (A) Synthetic oligomers used in the construction of the baculovirus gene transfer vectors and the gG-1- and gG-2-expressing recombinant baculoviruses. Oligoduplex AB was used in the construction of pPP-2, oligoduplex V78 for pAcDSM, oligoduplex SR34 for AcDSMgG-1, and oligoduplex D34 for AcDSMgG-2. Relevant restriction endonuclease sites are indicated. (B) Schematic representation of the method of inserting a foreign gene into the transfer vector pAcDSM. (C) Comparison of nucleotide sequences in the 5' nontranslated region of the wild-type baculovirus (AcNPV) and the recombinant viruses Ac373'gG-1 and AcDSMgG-1. Extraneous nucleotides relative to the wild-type polyhedrin sequence are boxed. The a preceding the initiation codon in Ac373'gG-1 was incorporated into the construct based on a published sequence (Hooft Van Iddekinge *et al.*, 1983) later found to be in error (Howard *et al.*, 1986).

J (pH1F-110, nucleotides 136,285 to 142,742) and HSV-2(G) *Bam*HI L (pH2G-112, nucleotides 2356 to 6894) fragments, which contain the intact gG-1 and gG-2 genes, respectively, were used as the starting point for engineering the genes for compatibility with the transfer vectors.

gG-1 insertion into pPP-2. pH1F-110 was digested with *Sph*I to remove nucleotides 137,617 to 142,742 of the HSV-1 fragment (removing two of the three *Nar*I sites in the insert) and ligated to itself. The resulting plasmid (pH1F-1001) was digested with *Nar*I and religated to itself, deleting nucleotides 136,285 to 136,749 and a small portion of the vector, resulting in pH1F-1002. This plasmid was digested with *Nar*I and *Hin*dIII, and the 873-bp fragment carrying the nearly complete gG-1 gene (nucleotides 136,749 to 137,622) was ligated to plasmid pPP-2, which had previously been digested with *Nar*I and *Hin*dIII. The resulting plasmid (pH1F-1011) was digested with *Eco*RI and *Hin*dIII. The fragment containing the modified gG-1 gene was purified from an agarose gel, made blunt-ended with T4 DNA polymerase, and ligated to pAc373, which had been digested with *Bam*HI and made blunt-ended by treatment with T4 DNA polymerase. A plasmid containing the gG-1 gene inserted in the proper orientation was designated pAc373'gG-1.

gG-1 insertion into pAcDSM. The 871-bp fragment between the *Nar*I and the *Sph*I sites of pH1F-1002 (nucleotides 136,749 to 137,620), carrying the nearly complete gG-1 gene, was ligated to pUC18 that had previously been digested with the same enzymes. The resulting plasmid, pSR1, was digested with *Nde*I and *Bam*HI, producing a 944-bp fragment that was ligated to pUC9 that had been previously digested with the same enzymes, resulting in pSR3. A synthetic oligoduplex, SR34 (Fig. 1A), was ligated between the *Ssp*I and the *Nar*I sites of pSR3. The resulting construct, plasmid pSR5, was successively incubated with *Kpn*I, T4 DNA polymerase, and *Bam*HI and ligated with the vector pAcDSM that had previously been successively reacted with *Pst*I, T4 DNA polymerase, and *Bam*HI. The resulting plasmid was designated pAcDSMgG-1.

gG-2 insertion in pAcDSM. pH2G-112 was digested with *Bam*HI and *Pvu*II to obtain a 3779-bp fragment (nucleotides 2356 to 6135) that was purified from agarose and ligated with pUC19 that had been digested with *Bam*HI and *Ssp*I. The resulting plasmid, pDS1, was digested with *Pst*I and *Hin*dIII and ligated with a synthetic oligodupex, D34 (Fig. 1A), to generate pDS2. The 1316-bp fragment (nucleotides 2515 to 3831) resulting from digesting pH2G-112 with *Hin*cII was purified from agarose and successively reacted with *Bst*NI,

T4 DNA polymerase, and *Sty*I. The resulting 161-bp fragment (nucleotides 2859 to 3020) was purified from agarose and then ligated to pDS2, which had previously been successively incubated with *Pst*I, T4 DNA polymerase, and *Sty*I. The resulting plasmid, pDS6, was digested with *Ssp*I and *Xho*I and ligated to an agarose-purified 2148-bp fragment obtained by digesting pH2G-112 with *Ssp*I and *Xho*I (nucleotides 2983 to 5131). The resulting plasmid, pDS7, was digested with *Hin*dIII and *Xho*I. The resulting 2311-bp fragment (carrying the complete gG-2 gene plus flanking plasmid-derived sequences) was ligated with pUC9 that had been digested with *Hin*dIII and *Sal*I. The resulting plasmid, pDS8, after successive reactions with *Kpn*I, T4 DNA polymerase, and *Bam*HI, was ligated with pAcDSM that had been successively reacted with *Pst*I, T4 DNA polymerase, and *Bam*HI to obtain pAcDSMgG-2.

Transfection and selection of recombinant baculoviruses

Procedures for transfection, selection of recombinant baculoviruses, and virus titration were performed as described (Summers and Smith, 1987). Recombinant viruses were plaque purified at least five times. Proper insertion of the transferred genes into the baculovirus genome was confirmed by blot hybridization analysis of the recombinant virus genomes (data not shown).

Protein analysis by immunoblots

Sf9 cells were grown either in Hink's medium supplemented with 10% fetal calf serum according to the method of Summers and Smith (1987) or in serum-free medium (Excell 400, J. R. Scientific, Woodland, CA) and infected with recombinant or wild-type baculoviruses at a multiplicity of infection of 10 PFU/cell in 25-cm^2 flasks at 27°. After 1.5 hr, the inoculum was removed and replaced with fresh medium. At the appropriate times postinfection, the cells were scraped from the flask and harvested by centrifugation for 10 min at 1500 *g* at 4°. The pellet was resuspended in disruption buffer (5.7 *M* urea, 2.8% SDS, and 1.8 *M* 2-mercaptoethanol), sonicated for 30 sec (output control 4, duty cycle 50%) in a cup horn sonicator (Model W-375, Heat Systems-Ultrasonic, Inc., Farmingdale, NY), and heated for 3 min at 95°. Proteins were separated by electrophoresis in polyacrylamide gels (Laemmli, 1970) (acrylamide to bis-acrylamide ratio of 37.5:1) and then electrically transferred onto nitrocellulose paper (BA85, Schleicher and Schuell) (Towbin *et al.*, 1979) using 0.1% SDS in the transfer buffer. Blots were incubated for 1 hr in blotto (5% skim milk, 0.01 *M* phosphate-buffered saline, pH 7.4, and 0.05% Tween 20) and then incubated for 1 hr in fresh blotto containing the appropriate dilution of either human serum or monoclonal antibody. After three 10-min washes in 0.05% Tween 20 in phosphate-buffered saline, blots were incubated with alkaline phosphatase-conjugated goat anti-human or alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Rockville Centre, NY) in 0.05% Tween 20 in phosphate-buffered saline for 2 hr, washed three times for 10 min with the same buffer, and developed with *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Bio-Rad) according to the vendor's protocol.

RESULTS

Vector construction

Two gene transfer vectors were constructed, pPP-2, which incorporates nucleotides −7 to −1 of the 5′ nontranslated leader sequence of the polyhedrin gene missing in the widely used transfer vector pAc373 (Smith *et al.*, 1985) but which results in recombinant baculoviruses containing 21 extraneous nucleotides in this region, and pAcDSM, which allows the construction of recombinant baculoviruses with the 5′ nontranslated leader sequence of the polyhedrin gene joined precisely to the coding region of the foreign gene at the translation initiation codon, with no missing or extraneous nucleotides. pPP-2 was constructed early in the course of these studies. It is awkward to use and offers few advantages relative to other currently available vectors. Its description is included here because it was used to construct the recombinant baculovirus Ac373′gG-1.

Construction of recombinant baculoviruses expressing gG-1 and gG-2

As described under Materials and Methods, recombinant baculoviruses Ac373′gG-1 and AcDSMgG-1 expressing gG-1 were constructed using pPP-2 in conjunction with pAc373 and pAcDSM, respectively. The nucleotide sequence in the vicinity of the translation initiation codon of these viruses is shown in Fig. 1C. A recombinant baculovirus expressing gG-2, AcDSMgG-2, was created using pAcDSM.

Synthesis and processing of baculovirus-expressed gG-1

Replica immunoblots of extracts from recombinant-infected, wild-type-infected, or uninfected Sf9 cells were reacted with either a human serum specimen that had been identified as HSV-1-positive and

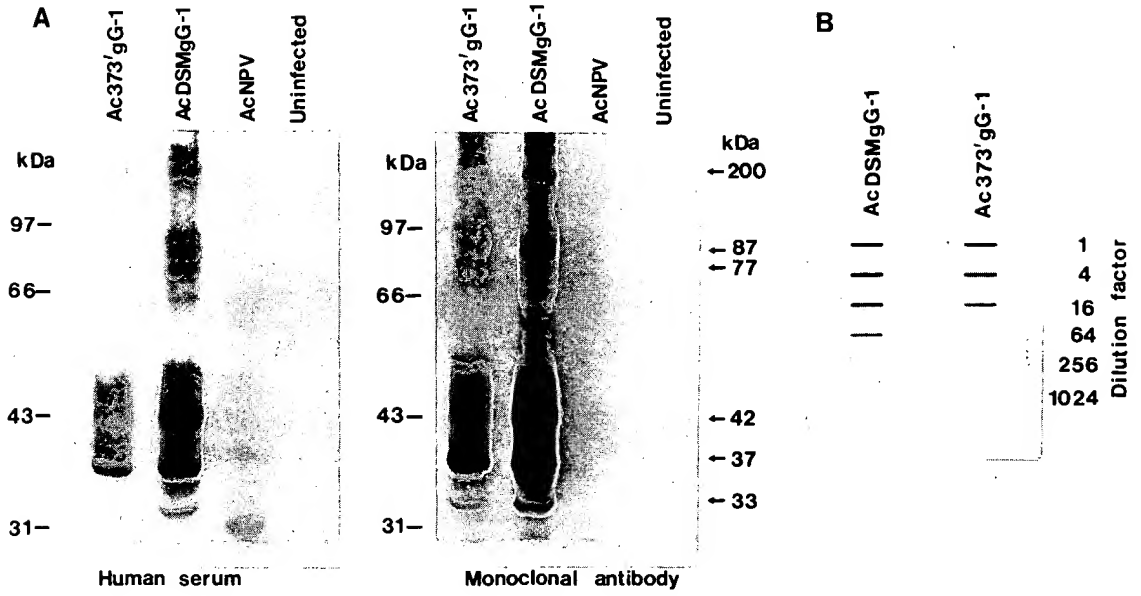

FIG. 2. Reaction of baculovirus-expressed gG-1s with antibodies. (A) Proteins extracted at 100 hr p.i. from equal numbers of Ac373'gG-1-, AcDSMgG-1-, and AcNPV-infected or uninfected Sf9 cells were separated by SDS–PAGE in 11% gels, transferred to nitrocellulose membranes, and then tested with the indicated antibodies. The positions of the molecular mass standards are shown on the side of each panel, (phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; and carbonic anhydrase, 31 kDa). The apparent molecular mass of gG-1-related species (arrows) is indicated. (B) Quantitative comparison of the amount of gG-1 expressed in Sf9 cells by the recombinants Ac373'gG-1 and AcDSMgG-1. Cell extracts similar to those used in A were fourfold serially diluted in 0.01 *M* phosphate-buffered saline, pH 7.4, bound to a nitrocellulose membrane using a slot-blot apparatus, and reacted with gG-1-specific monoclonal antibody (H1379).

HSV-2-negative using an HSV type-specific indirect hemagglutination assay (IHA) (Bernstein and Stewart, 1971) or a monoclonal antibody specific for gG-1 (H1379) (Lee *et al.*, 1986) (Fig. 2A). None of the antibodies reacted with proteins in the lanes containing proteins from AcNPV-infected or uninfected cells, except for a weak reaction between some human serum specimens and polyhedrin in AcNPV-infected cells. The pattern of reactivity with both antibodies was identical in the lanes containing the gG-1 recombinant-infected cell extracts (lanes Ac373'gG-1 and AcDSMgG-1). The major reacting bands appeared at 37 and 42 kDa apparent molecular mass (37K and 42K) within a region of diffuse reactivity between 36 and 48 kDa apparent molecular mass.

Expression of the recombinant gG-1s differed in two respects. (i) The intensity of the reaction with both antibodies was higher in extracts of cells infected with AcDSMgG-1 than with Ac373'gG-1 (Fig. 2A). (ii) In extracts of Sf9 cells infected with AcDSMgG-1, 42K reacted more than 37K. In extracts of Sf9 cells infected with Ac373'gG-1, the opposite was true, with 42K being very faint.

Slot-blot analysis was used to eliminate difficulties inherent in quantifying multiple diffuse bands in electropherograms. The intensity of the reaction was about fourfold greater in extracts harvested from cells infected with AcDSMgG-1 than with Ac373'gG-1 at either 100 hr p.i. (Fig. 2B) or 72 hr p.i. (data not shown). Thus more gG-1 was expressed from the construct that mimicked the polyhedrin 5' nontranslated leader sequence (AcDSMgG-1) than from the construct that contained 21 extraneous nucleotides in this region (Ac373'gG-1).

We monitored the levels of gG-1 production and the ratios of intensity between the two major gG-1 bands in the two recombinants as a function of time after infection (Fig. 3A). The major gG-1 bands (37K and 42K) were first detected at 36 hr p.i. At all time points, more gG-1 was detected in the lanes containing extracts from cells infected with AcDSMgG-1 (Fig. 3A, lanes b). The maximum expression with both recombinants occurred at about 72 hr p.i. As was previously seen at 100 hr p.i., at all time points 42K was more abundant than 37K in AcDSMgG-1-infected cells (lanes b), while in Ac373'gG-1-infected cells (lanes a), 37K was more abundant than 42K. This indicates that the difference in processing of gG-1 may be due to differences between the transfer vectors. Similar patterns and levels of synthesis were observed in two independently plaque-purified progeny of the transfection that produced Ac373'gG-1 (data not shown).

Treatment of AcDSMgG-1-infected Sf9 cells with tunicamycin, an inhibitor of a precursor necessary for

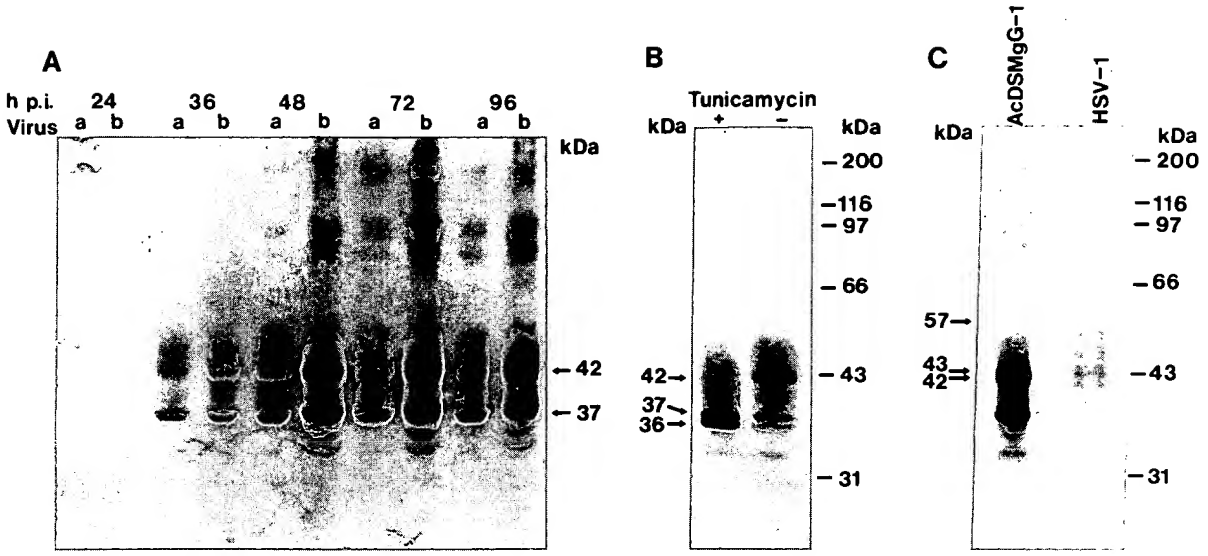

FIG. 3. Synthesis and processing of baculovirus-expressed gG-1. (A) Time course of the synthesis of gG-1 in Ac373'gG-1-infected and AcDSMgG-1-infected (lanes a and b, respectively) Sf9 cells. Cells were harvested at the indicated times, treated as those in Fig. 2A, and reacted with a HSV-1-positive human serum specimen. (B) Immunoblot of proteins extracted from Sf9 cells infected with AcDSMgG-1 and grown in the presence (+) or the absence (−) of 3 μg/ml tunicamycin from 24 hr p.i. until 54 hr p.i. Blots were reacted with gG-1-specific monoclonal antibody (H1379). (C) Immunoblot analysis of proteins extracted from Sf9 cells infected with AcDSMgG-1 and from HEp-2 cells infected with HSV-1(F); about 100-fold more infected cells of the latter were used. Myosin (200 kDa) and β-galactosidase (116.3 kDa) were used in addition to the molecular mass standards used in Fig. 2. The apparent molecular mass of bands discussed in the text (arrows) is indicated.

N-linked glycosylation (Hemming, 1982), resulted in a great reduction in the intensity of all the bands over 37 kDa apparent molecular mass, most prominently 42K, and an increase in the intensity of an otherwise very faint species of 36 kDa apparent molecular mass (36K) (Fig. 3B). The abundant 37K is of similar intensity in infected cells cultured either in the presence or in the absence of tunicamycin. In heavily loaded gels, species of 77 and 87 kDa apparent molecular mass (77K and 87K) were replaced by species of 74 and 83 kDa (74K and 83K) apparent molecular mass (data not shown).

In a comparison of baculovirus- and HSV-1-expressed gG-1, major bands with apparent molecular masses of 42 and 43 kDa (42K and 43K) and a smear between 50 and 57 kDa apparent molecular mass were found in extracts of HEp-2 cells infected with HSV-1(F) (Fig. 3C). In heavily loaded gels, the smear extended to 66 kDa apparent molecular mass (not shown).

Synthesis and processing of baculovirus-expressed gG-2

Replica immunoblots of extracts of Sf9 cells infected with AcDSMgG-2, wild-type baculovirus, or uninfected were reacted with either a HSV-2-positive and a HSV-1-negative human serum specimen or with a gG-2-specific monoclonal antibody (H1206) (Lee *et al.*, 1985) (Fig. 4A). In lanes containing extracts from AcDSMgG-2-infected cells, both antibodies reacted with bands with apparent molecular masses of 107, 118, 128, and 143 kDa (107K, 118K, 128K, and 143K). The human serum specimen reacted most strongly with 128K, and the monoclonal antibody with 118K. This difference in reactivity between the human serum and the monoclonal antibody is not a general phenomenon, inasmuch as other human serum specimens reacted most strongly with 118K (data not shown). In overloaded gels a weakly reactive protein with an apparent molecular mass of 34 kDa (34K) was detected.

We monitored expression of gG-2 in recombinant baculovirus-infected cells as a function of time after infection (Fig. 4B). Only one band (118K, open triangle) was detected at the earliest time point examined (24 hr p.i.). By 36 hr p.i. it had increased in intensity and two faint bands appeared (107K and 128K, solid triangles). From 48 hr p.i. onward, the four bands (107K, 118K, 128K, and 143K) previously seen at 100 hr p.i. (Fig. 4A) were visible, with the maximum accumulation of protein at 72 hr p.i. The weakly reactive 34K protein was first detected at 36 hr p.i.

In the electrophoretic pattern of extracts of Sf9 cells infected with AcDSMgG-2 and treated with tunicamycin, bands migrating with apparent molecular masses of 105, 110, and 120 kDa (105K, 110K, and 120K) were

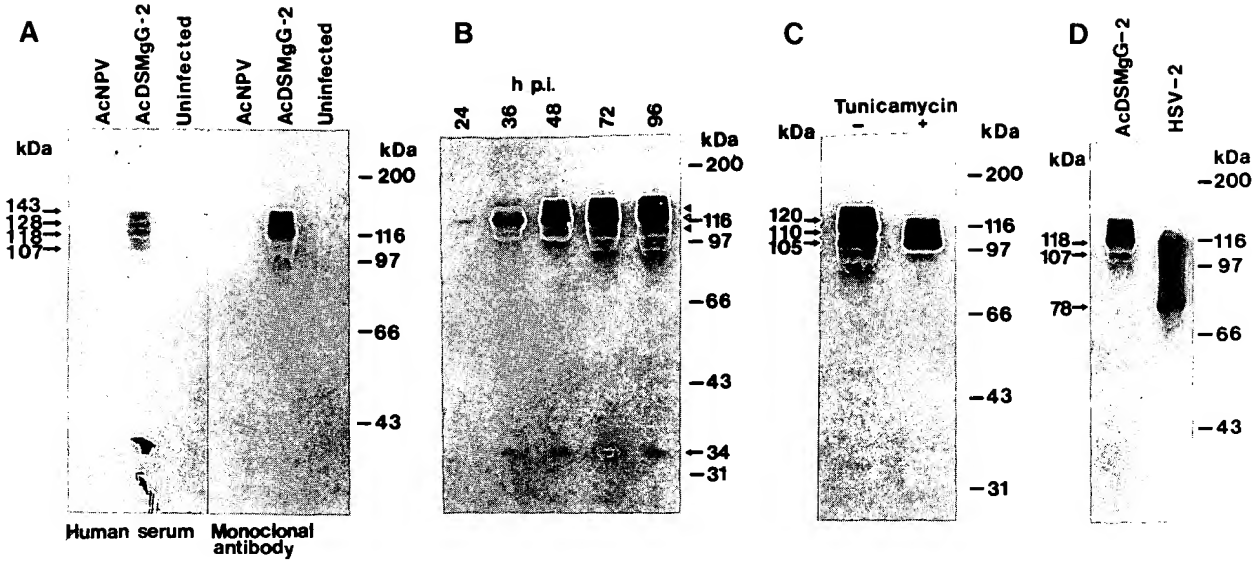

FIG. 4. Synthesis and processing of baculovirus-expressed gG-2. (A) Proteins extracted at 100 hr p.i. were separated in a 9% SDS–PAGE gel, then transferred to nitrocellulose, and reacted with the indicated antibodies. (B) Time course of the synthesis of gG-2 in AcDSMgG-2-infected Sf9 cells. Proteins extracted from cells harvested at the times indicated were treated as those in A and then reacted with gG-2-specific monoclonal antibody (H1206). (C) Immunoblot of proteins extracted from Sf9 cells infected with AcDSMgG-2 and grown in the presence (+) or the absence (−) of 3 μg/ml tunicamycin from 24 to 54 hr p.i. Blots were reacted with gG-2-specific monoclonal antibody (H1206). (D) Immunoblot analysis of proteins extracted from Sf9 cells infected with AcDSMgG-2 and from HEp-2 cells infected with HSV-2(G); about 1.8-fold more infected cells of the latter were used. Molecular mass standards were the same as those for Fig. 3. The apparent molecular mass of bands discussed in the text (arrows or triangles) is indicated.

present, whereas in untreated cells, the 107K, 118K, 128K, and 143K species were seen (Fig. 4C). In overloaded gels, a band migrating with an apparent molecular mass of 30 kDa was present in the extract of infected cells treated with tunicamycin, in contrast with the 34K species seen in untreated cells (data not shown).

In comparisons of baculovirus- and HSV-2-expressed gG-2, in the lane containing extracts from HEp-2 cells infected with HSV-2(G) (Fig. 4D), a smear of reactivity was seen ranging from an apparent molecular mass of 78 through 118 kDa with distinctive species at 78, 107, and 118 kDa apparent molecular mass. In overloaded gels, a faint band with apparent molecular mass of 36 kDa was detected (data not shown).

HSV type specificity of the reaction of human serum specimens with the baculovirus-expressed proteins

Proteins in extracts of Sf9 cells expressing the recombinant gG-1 or gG-2 were tested for reactivity with 10 different human serum specimens previously characterized by using an HSV type-specific IHA (Bernstein and Stewart, 1971). As a representative example, patterns obtained with 3 of these 10 specimens are shown in Fig. 5. Using the gG-1 37K and 42K species and the gG-2 118K species, plus either or both of the 128K and 143K species, as markers of HSV-1 and HSV-2 type-specific reaction, respectively, a serum specimen positive for HSV-1 and negative for HSV-2 by IHA, and a serum specimen positive for HSV-2 and negative for HSV-1 by IHA, each reacted in a type-specific manner in the immunoblot assay (Figs. 5A and 5B, respectively). A serum specimen weakly positive for both types of antibodies by IHA reacted with the gG-1-specific 37K and 42K species (empty triangles) in the lane of the recombinant gG-1, and reacted weakly but clearly with the gG-2 118K and 128K species (full triangles) in the lane containing recombinant gG-2 (Fig. 5C). We have seen various patterns of weak reactivity between human serum specimens and extracts of wild-type baculovirus-infected Sf9 cells as well as uninfected cells. The faint bands seen at 36 kDa apparent molecular mass in the gG-2 lanes of Fig. 5, as well as the bands seen at 170 kDa apparent molecular mass in Fig. 5C, are examples of these reactions. In no case was the reactivity of an extent or nature to lead to ambiguity in interpretation. Results obtained with the other seven serum specimens were in agreement with the IHA results (data not shown).

DISCUSSION

We constructed a transfer vector, pAcDSM, that facilitates the construction of recombinant baculovir-

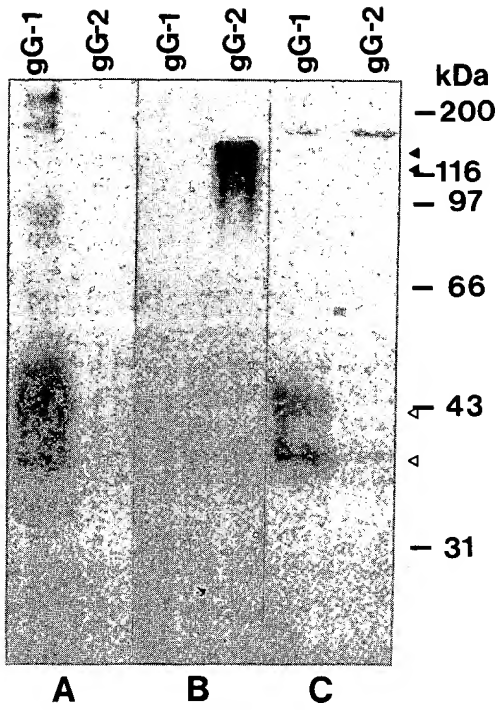

FIG. 5. Immunoblot analysis of the HSV type specificity of the reaction of human serum specimens with AcDSMgG-1- and AcDSMgG-2-infected Sf9 cell extracts. Proteins were separated by SDS–PAGE in a 11% gel, transferred to nitrocellulose, and then reacted with serum specimens known to be HSV-1-positive and HSV-2-negative (A), HSV-1-negative and HSV-2-positive (B), and having a low positive titer to both HSV-1 and HSV-2 (C). Bands considered to be diagnostic for HSV-1-specific reactivity are indicated with open triangles, and those for HSV-2 with solid triangles. Molecular mass standards were the same as those for Fig. 3.

uses containing no missing or extraneous nucleotides in the region 5′ to the translation initiation codon. No other vector reported to date allows such constructs. Although we did not compare the performance of this vector with that of recently described vectors (Matsuura *et al.,* 1987; Luckow and Summers, 1989) which more closely, albeit imperfectly, mimic the gene expression control environment of the native polyhedrin gene than earlier transfer vectors (Smith *et al.,* 1985), we did observe fourfold greater expression of gG-1 from pAcDSM than from a construct that contains 21 extraneous nucleotides in the 5′ nontranslated leader sequence. The nature of pAcDSM will allow careful dissection of the environment in the vicinity of the translation initiation codon through the construction of viruses with precise sequence modifications in this region.

Unexpectedly, in addition to the difference in the level of expression between the two gG-1-expressing recombinants, we observed a difference in the ratio of nonglycosylated precursor (37K, see below) to glycosylated product (42K), with gG-1 expressed from AcDSMgG-1 being the most efficiently processed. Inasmuch as the gG-1 coding sequence was not altered during the construction of the recombinants, these results indicate an effect of mRNA structure on protein processing efficiency. Further studies will be required to test this hypothesis.

Biosynthesis of gG-1

A scenario for the biosynthesis of gG-1 in baculovirus-infected Sf9 cells that accounts for our results is as follows: The 37K species is insensitive to tunicamycin and is likely to be the primary nonglycosylated translation product with its signal peptide uncleaved, indicating that it was not translocated to the lumen of the rough endoplasmic reticulum. The 36K species is most abundant during growth in the presence of tunicamycin and would be the nonglycosylated translation product without its signal peptide. The broad band produced by 42K, which is sensitive to tunicamycin, would be the translation product after having its signal peptide removed and being N-linked glycosylated. Species 77K, 87K, and 200K are likely to be products of further processing. In heavily loaded gels, 77K and 87K were replaced by 74K and 83K species upon treatment with tunicamycin (data not shown), indicating that the generation of the low mobility gG-1-reactive molecules is not dependent on N-linked glycosylation. The low mobility gG-reactive polypeptides are not likely to represent gG oligomers, because cell lysates were boiled in the presence of a detergent, a reducing agent, and urea prior to electrophoresis. The low abundance of the 33K species is likely to be the result of proteolytic degradation.

The biosynthesis of gG-1 in baculovirus-infected insect cells differs in several respects from its synthesis in mammalian cells infected with HSV-1- or gG-1-expressing vaccinia virus recombinants. In a direct comparison of baculovirus- and HSV-1-expressed gG-1, we observed products with apparent molecular masses of 42 and 43 kDa in HSV-1-infected cells which comigrated with 42K. It is possible that the 42- and 43-kDa species found in HSV-1-infected cells correspond to the 44- to 48-kDa species reported by others (Ackermann *et al.,* 1986) using the same strain of virus (strain F) and the same monoclonal antibody (H1379). However, the relationship between these products and the closely migrating products found in the baculovirus-infected cells is not clear. The baculovirus-expressed protein is tunicamycin sensitive and thus glycosylated, as is a product of similar size found in cells infected with a vaccinia virus/gG-1 recombinant (Sullivan and Smith, 1987), while the HSV-1 product is poorly labeled with glucosamine (Ackermann *et al.,* 1986). In addition,

a similarly sized product is synthesized in the presence of tunicamycin in HSV-1 strain HFEM-infected cells (Richman *et al.*, 1986).

Biosynthesis of gG-2

A scenario for the biosynthesis of gG-2 in baculovirus-infected Sf9 cells is as follows: 107K is the primary translation product including the signal peptide. This is based on two observations. (i) It is synthesized in the absence of tunicamycin. (ii) Since no species migrating faster than 105K were detected in cells infected in the presence of tunicamycin, 107K is inferred to not be N-linked glycosylated, is therefore likely to be unaffected by tunicamycin treatment, and is probably obscured by the other species in the gel. The 105K species is the primary translation product after cleavage of its signal peptide based on its increase in abundance in the presence of tunicamycin. The tunicamycin-sensitive 118K is the cleaved primary translation product after N-linked glycosylation. Species 128K and 143K, and 110K and 120K, synthesized in the absence or presence, respectively, of tunicamycin are possibly the products of O-linked glycosylation. The tunicamycin-sensitive 34K is likely to be the result of a proteolytic degradation.

As with gG-1, there were significant differences between the biosynthesis of gG-2 in baculovirus-infected insect cells and that in HSV-2-infected mammalian cells. In both baculovirus- and HSV-2-infected cells a tunicamycin-sensitive product of 118-kDa apparent molecular mass is synthesized. In baculovirus-infected cells, it appears that this product is processed further into higher molecular mass forms, but in HSV-2-infected cells this product is cleaved to generate species of 31 kDa (31K) (Su *et al.*, 1987) and 74 kDa (74K) (Balachandran and Hutt-Fletcher, 1985) apparent molecular mass. The 74K species is subsequently O-linked glycosylated yielding a species of 105 kDa apparent molecular mass (Balachandran and Hutt-Fletcher, 1985). The 31K species is further glycosylated to a species of 34-kDa apparent molecular mass, which is efficiently secreted from infected cells, and it is not detected by the same monoclonal antibody that detects the higher mobility products (Su *et al.*, 1987). This 34-kDa apparent molecular mass HSV-2-infected cell species differs from the similarly sized product we observed in recombinant baculovirus-infected cells, in that the baculovirus product reacts with the same monoclonal antibody as do the lower mobility products.

HSV type specificity of baculovirus expressed gG

The several differences in the synthesis and processing of gG-1 and gG-2 in insect cells, relative to that observed here and by others during infections of mammalian cells with HSV, reflect differences in protein processing mechanisms between insect and mammalian cells. Despite these differences, the recombinant proteins were recognized in a HSV type-specific manner by the 10 human serum specimens tested here. To further test the use of the baculovirus expressed proteins as substrates for serologic tests we are currently testing a set of over 80 serum specimens that had previously been characterized using gG immunodot assays (Lee *et al.*, 1985, 1986; Nahmias *et al.*, 1986). Preliminary results confirm the type specificity of baculovirus-expressed gG-1 and gG-2 (Sánchez-Martínez *et al.*, unpublished data).

ACKNOWLEDGMENTS

We thank Martha Kay Personnette for technical assistance, John Stewart for the well-characterized human sera, Brian P. Holloway for the synthesis of the oligomers, and Carlos Lopez for helping initiate this work.

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